Acquired resistance to macrolides in *Pseudomonas aeruginosa* from cystic fibrosis patients

Muhammad-Hariri Mustafa[1,2], Shaunak Khandekar[1], Michael M. Tunney[3], J. Stuart Elborn[3], Barbara C. Kahl[4], Olivier Denis[5], Patrick Plésiat[6], Hamidou Traore[2], Paul M. Tulkens[1], Francis Vanderbist[2] and Françoise Van Bambeke[1]

Affiliations: [1]Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium. [2]SMB Laboratories, Brussels, Belgium. [3]Queen's University of Belfast, Belfast, UK. [4]University Hospital Münster, Münster, Germany. [5]Hôpital Erasme, Université libre de Bruxelles, Brussels, Belgium. [6]Laboratoire de Bactériologie, Hôpital Jean Minjoz, Besançon, France.

Correspondence: Françoise Van Bambeke, Pharmacologie cellulaire et moléculaire, Louvain Drug Research Institute, Université catholique de Louvain, Avenue Mounier 73, B1.73.05, 1200 Brussels, Belgium.
E-mail: francoise.vanbambeke@uclouvain.be

@ERSpublications
Ribosomal mutations are frequent in *P. aeruginosa* from cystic fibrosis patients, owing to chronic oral macrolide use http://ow.ly/B5vC308qfjl

Cite this article as: Mustafa M-H, Khandekar S, Tunney MM, *et al*. Acquired resistance to macrolides in *Pseudomonas aeruginosa* from cystic fibrosis patients. *Eur Respir J* 2017; 49: 1601847 [https://doi.org/10.1183/13993003.01847-2016].

ABSTRACT Cystic fibrosis (CF) patients receive chronic treatment with macrolides for their antivirulence and anti-inflammatory properties. We, however, previously showed that *Pseudomonas aeruginosa*, considered as naturally resistant to macrolides, becomes susceptible when tested in a eukaryotic medium rather than a conventional broth.

We therefore looked for specific macrolide resistance determinants in 333 CF isolates from four European CF centres in comparison with 48 isolates from patients suffering from hospital-acquired pneumonia (HAP).

Minimum inhibitory concentrations (MICs) of macrolides and ketolides measured in eukaryotic medium (RPMI-1640) were higher towards CF than HAP isolates. Gene sequencing revealed mutations at three positions (2045, 2046 and 2598) in domain V of 23S rRNA of 43% of sequenced CF isolates, but none in HAP isolates. Enzymes degrading extracellular polymeric substances also reduced MICs, highlighting a role of the mucoid, biofilm-forming phenotype in resistance. An association between high MICs and chronic azithromycin administration was evidenced, which was statistically significant for patients infected by the Liverpool Epidemic Strain.

Thus, ribosomal mutations are highly prevalent in CF isolates and may spread in epidemic clones, arguing for prudent use of oral macrolides in these patients. Measuring MICs in RPMI-1640 could be easily implemented in microbiology laboratories to phenotypically detect resistance.

This article has supplementary material available from erj.ersjournals.com

Received: Sept 20 2016 | Accepted after revision: Jan 15 2017

Support statement: This work was supported by the Doctiris programme of Innoviris (Région Bruxelloise, Belgium). M-H. Mustafa is a doctoral fellow from the Doctiris programme and F. Van Bambeke is Maître de Recherches of the Belgian Fonds National de la Recherche Scientifique. Cempra Pharmaceuticals (Chapel Hill, NC, USA) provided solithromycin. Funding information for this article has been deposited with the Open Funder Registry.

Conflict of interest: Disclosures can be found alongside this article at erj.ersjournals.com

Introduction

The leading cause of morbidity and mortality in cystic fibrosis (CF) patients is progressive respiratory dysfunction, partially due to chronic bacterial infections. *Pseudomonas aeruginosa* is among the most frequent pathogens in the respiratory tract of CF adults and causes irreversible lung damage [1]. These patients therefore receive repetitive and prolonged treatments with antipseudomonal antibiotics. Many CF patients also receive long-term treatment with macrolides, based on their known antivirulence [2] and anti-inflammatory [3] properties, which improve their respiratory function [4] particularly when chronically infected with *P. aeruginosa.* Macrolides are considered intrinsically inactive against *P. aeruginosa*, with high ($\geqslant$256 mg·L^{-1}) minimal inhibitory concentrations (MICs) measured in the recommended conventional broth [5]. This intrinsic resistance is due to the efflux of macrolides out of the bacteria *via* multidrug efflux systems [6], among which MexAB–OprM and MexXY–OprM are constitutively expressed in wild-type isolates [7]. Yet, macrolides show low MICs against *P. aeruginosa* when tested in the presence of serum, bronchoalveolar lavage fluid or culture media used for eukaryotic cell cultures. Enhanced macrolide activity in these media results from an increased permeability of the bacterial outer membrane [8]. This favours the diffusion of macrolides inside the bacteria, where they repress the expression of OprM, the outer membrane porin coupled to MexAB and MexXY efflux transporters (responsible for macrolide efflux), further increasing the macrolide intrabacterial concentration to a level where clinically meaningful activity can be observed [8].

Whether macrolides also show activity against *P. aeruginosa in vivo* and thereby contribute to the improvement of lung function in infected CF patients remains to be established. Nevertheless, the extensive use of macrolides in this population clearly raises the question of the potential risk of selecting macrolide-resistant *P. aeruginosa*, thereby decreasing their potential usefulness in CF patients and increasing the global resistance burden.

While macrolide resistance is well described in Gram-positive bacteria (due to target modification (methylation or mutation in domain V of 23S rRNA) and/or active efflux [9, 10]), a single publication reports resistance associated with mutations in domain V of 23S rRNA of six *P. aeruginosa* isolates from CF patients treated by macrolides [11]. The extent of this problem is thus largely unknown.

In this study, we determined macrolide activity in a collection of 333 *P. aeruginosa* isolates from CF patients, using eukaryotic cell culture medium (RPMI-1640) in order to detect increases in MICs that could be ascribed to the acquisition of resistance mechanisms (which would have escaped detection if using conventional broth). We included in our analysis 48 isolates collected from intensive care patients suffering from hospital-acquired pneumonia (HAP) [12], in whom chronic exposure to macrolides could be excluded. We compared conventional macrolides (azithromycin and clarithromycin) to ketolides, the next-generation macrolides that are less affected by ribosomal mutations at domain V of 23S rRNA, as they also bind to ribosome domain II [13, 14]. Among ketolides, we used telithromycin (registered in 2001 but restricted use due to safety issues) and solithromycin (a new fluoroketolide [15] that successfully completed phase III clinical trials for community-acquired pneumonia) [16, 17].

In brief, we found that CF isolates were significantly less susceptible to macrolides than HAP isolates, with about half of them harbouring mutations in domain V of their 23S rRNA, while no mutations were observed in HAP isolates. MICs of ketolides (especially solithromycin) were less affected by these mutations. Thus, the data document a so-far unreported development of resistance to macrolides in *P. aeruginosa* collected from CF patients and argue for prudent use of macrolides in this population.

Materials and methods

P. aeruginosa

The common laboratory strain *P. aeruginosa* PAO1 was used as a reference and strain ATCC 27853 as quality control for susceptibility tests. PAO1-pMES-23S(A2045G) is a PAO1 derivative containing a plasmid pME6031 encoding the whole rRNA operon with an A2045G mutation in domain V of 23S rRNA [11].

Clinical isolates (n=333) were randomly collected from 155 patients (aged 1–59 years) in four European CF centres (Hôpital des enfants malades Reine Fabiola/Hôpital Erasme, Brussels, Belgium (n=88); Hôpital Jean Minjoz, Besançon, France (n=80); Universitätsklinikum Münster, Münster, Germany (n=66); Queen's University of Belfast, Belfast, UK (n=99)) during routine visits. 48 isolates collected from intensive care patients suffering from HAP at the Hôpital Erasme [12] were used for comparison. For UK isolates, those belonging to the Liverpool Epidemic Strain (LES) [18] were previously identified by multilocus sequence typing [19]. Data on file for UK and German isolates also stipulated whether patients were receiving azithromycin regularly (500 mg three times per week continuously in the UK or for intermittent 6-month periods in Germany).

Antibiotics

Antibiotics were obtained as microbiological standards: clarithromycin and azithromycin (Teva, Petach Tikva, Israel), telithromycin (Sanofi-Aventis, Romainville, France), and solithromycin (Cempra Pharmaceuticals, Chapel Hill, NC, USA).

Susceptibility testing

MICs were determined by broth microdilution in cation-adjusted Mueller–Hinton broth (CA-MHB; BD Bioscience, Franklin Lakes, NJ, USA) [5] and RPMI-1640 (Invitrogen, Paisley, UK) adjusted to pH 7.4 and complemented with 10% fetal calf serum [8]. MICs were also measured in the presence of 1) 20 $mg{\cdot}L^{-1}$ Phe-Arg-β-naphthylamide (PAβN; a broad-spectrum efflux pump inhibitor) and 1 mM $MgSO_4$ (to strengthen the outer membrane and thereby limit PAβN toxicity [20]), or 2) 20 $U{\cdot}mL^{-1}$ alginate lyase, 0.02% DNase and 20 mM $MgCl_2$ (all from Sigma-Aldrich, St Louis, MO, USA), or 100 $mg{\cdot}L^{-1}$ Proteinase K (Thermo Fisher, Waltham, MA, USA).

Assessment of outer membrane permeability

Bacteria were incubated in CA-MHB or RPMI-1640 for 4 h, after which 25 µM 1-*N*-phenylnaphthylamine was added [8]. Fluorescence was read using a Spectramax (Molecular Devices, Sunnyvale, CA, USA) microplate reader ($\lambda_{excitation}/\lambda_{emission}$ 355/405 nm). Full permeabilisation was achieved by adding 3% Triton X-100 [21] 45 min before reading.

Gene sequencing of rRNA and proteins

The genes encoding domains II and V of the 23S ribosomal subunit or the entire ribosomal proteins L4 and L22 were amplified by PCR (see supplementary table S1 for primers; Eurogentec, Seraing, Belgium) and sequenced (Beckman-Coulter Genomics, Takeley, UK). For domain V, we focused on the 625-bp region where mutations have been previously described (A2058G, A2059G and C2611T [22] in the 23S subunit of *Escherichia coli* [23], corresponding to positions 2045, 2046 and 2598 in *Pseudomonas*).

Plasmid construction and transformation in PAO1

Plasmid PAO1-pMES-23S(A2045G) was extracted from its host strain using a GeneJET Plasmid Miniprep Kit (Thermo Fisher) and the gene encoding 23S rRNA was reverted back to its wild-type sequence using a Q5 Site-Directed Mutagenesis Kit (NEB, Ipswich, MA, USA), resulting in plasmid pMES-23S(0). This plasmid was used as a template to introduce each of the point mutations observed in the 23S RNA gene (table 1) and the resulting vectors were transformed into PAO1.

Statistical analyses

Statistical analyses were performed using Prism version 7.01 (GraphPad, San Diego, CA, USA).

Ethics

According to a decision of the Ethical Committee of the Université catholique de Louvain, the investigations did not fall under the scope of the law on human experimentation as all isolates had been collected during routine sampling and patients' data had been anonymised before being transmitted to the investigators.

Results

Susceptibility of CF and HAP isolates to macrolides and ketolides

Figure 1 shows the MIC distributions of clarithromycin (figure 1a and d) and azithromycin (figure 1b and e) as measured in CA-MHB and RPMI-1640 for CF *versus* HAP isolates. As previously described [8], MICs were one to three dilutions lower in RPMI-1640 than in CA-MHB. RPMI-1640 is therefore useful to observe differences in activity among antibiotics and/or isolates. Azithromycin was slightly more active than clarithromycin. HAP isolates were more susceptible than CF isolates, the difference being most apparent for azithromycin in RPMI-1640. All further experiments were performed on a subset of the CF collection selected to cover the whole range of azithromycin MICs. The two conventional macrolides were first compared with two ketolides. Figure 1c and f show the MIC distributions of solithromycin (compared with azithromycin for the same selected CF isolates in figure 1c), with the MIC_{50}/MIC_{90} of clarithromycin, azithromycin, telithromycin and solithromycin shown in the accompanying table. Ketolides were more potent than macrolides in both media and for both CF and HAP isolates, with solithromycin MIC_{50}/MIC_{90} being 1–2 $\log_2$ dilutions lower than those of telithromycin against CF isolates (in both media) and HAP isolates (in CA-MHB). Yet, CF isolates remained less susceptible to ketolides than HAP isolates.

TABLE 1 MICs of macrolides and ketolides in cation-adjusted Mueller–Hinton broth (CA-MHB) and RPMI-1640 for *Pseudomonas aeruginosa* PAO1, transformed PAO1 and cystic fibrosis isolates with ribosomal mutations, classified according to the type of mutation detected

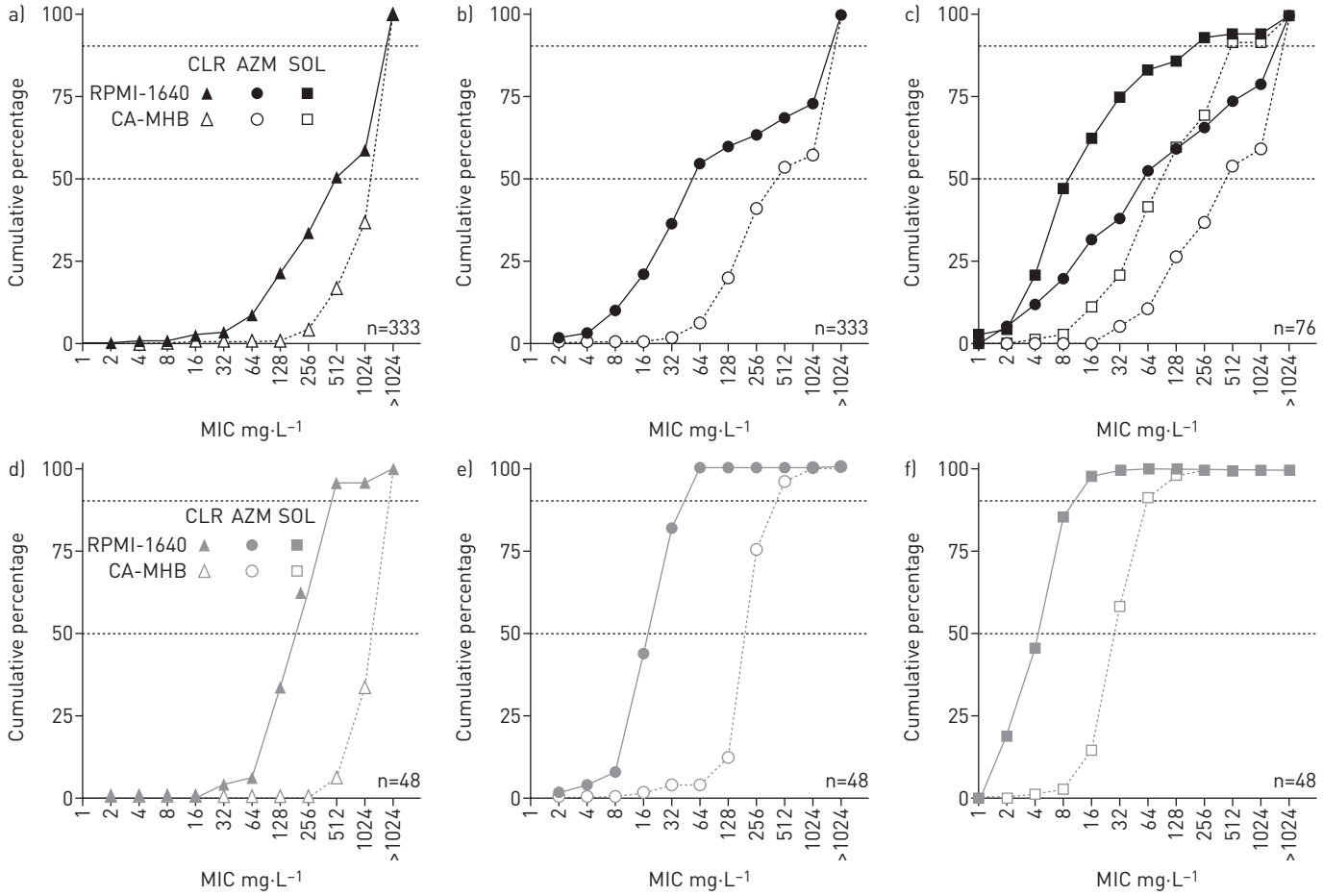
Strain	Patient ID	Country	Azithromycin treatment	Mutated alleles n	MIC mg·L^{-1}							
					Clarithromycin		Azithromycin		Telithromycin		Solithromycin	
					CA-MHB	RPMI-1640	CA-MHB	RPMI-1640	CA-MHB	RPMI-1640	CA-MHB	RPMI-1640
PAO1					512	256	128	32	64	32	32	16
PAO1-pMES-23S(0)					512	256	128	32	64	32	32	16
Mutation: A2045G												
Control strain: PAO1-pMES-23S(A2045G)					>1024	>1024	>1024	1024	64	32	32	16
2162	BA1	France	No data	3	>1024	>1024	>1024	>1024	1024	1024	512	256
2964	BV1	France	No data	3	>1024	>1024	>1024	>1024	1024	1024	512	512
3066		France	No data	3	>1024	>1024	>1024	>1024	1024	1024	512	256
154-1	154	Germany	Yes	3	>1024	>1024	>1024	>1024	>1024	256	>512	64
186	186	Germany	Yes	3	>1024	>1024	>1024	512	1024	16	512	8
195-2	195	Germany	No	3	>1024	>1024	>1024	512	1024	1024	512	256
CF5	AW	UK (LES)	Yes	4	>1024	>1024	>1024	>1024	>1024	>1024	>512	>512
BM6	AD	UK (LES)	No	4	>1024	>1024	>1024	>1024	>1024	>1024	>512	256
CF12	AON	UK (LES)	No	4	>1024	>1024	>1024	>1024	>1024	>1024	>512	128
Mutation: A2045T												
Control strain: PAO1-pMES-23S(A2045T)					>1024	>1024	>1024	1024	64	32	32	16
BF4	GM	UK	Yes	4	>1024	>1024	>1024	>1024	>1024	>1024	>512	>512
Mutation: A2046G												
Control strain: PAO1-pMES-23S(A2046G)					>1024	>1024	>1024	1024	64	32	32	16
2751	WL1	France	No data	2	>1024	>1024	>1024	256	1024	256	512	128
2801	PS1	France	No data	4	>1024	>1024	>1024	>1024	1024	1024	512	256
127-2	127	Germany	No	4	>1024	>1024	>1024	>1024	>1024	>1024	>512	>512
129-8	129	Germany	No	4	>1024	>1024	>1024	1024	1024	64	512	64
134-2	134	Germany	Yes	3	>1024	>1024	>1024	1024	1024	1024	512	256
205	205	Germany	No	3	>1024	>1024	>1024	>1024	>1024	>1024	>512	>512
CF15	CT	UK (LES)	Yes	4	>1024	>1024	>1024	>1024	>1024	>1024	>512	>512
CF22	GD	UK (LES)	Yes	1	>1024	1024	512	128	512	64	128	32
CF45	ML	UK (LES)	Yes	4	>1024	>1024	>1024	>1024	>1024	>1024	>512	>512
Mutation: A2046T												
Control strain: PAO1-pMES-23S(A2046T)					>1024	>1024	>1024	1024	64	32	32	16
132-2	132	Germany	Yes	4	>1024	>1024	>1024	>1024	>1024	>1024	>512	8
172-3	172	Germany	No	3	>1024	>1024	>1024	>1024	>1024	1024	256	8

Continued

TABLE 1 Continued

Strain	Patient ID	Country	Azithromycin treatment	Mutated alleles n	MIC mg·L^{-1}							
					Clarithromycin		Azithromycin		Telithromycin		Solithromycin	
					CA-MHB	RPMI-1640	CA-MHB	RPMI-1640	CA-MHB	RPMI-1640	CA-MHB	RPMI-1640
Mutation: C2598T												
Control strain: PAO1-pMES-23S(C2598T)					>1024	1024	512	256	64	32	32	16
2036	PS1	France	No data	3	>1024	>1024	>1024	>1024	256	64	64	8
5637	RS1	France	No data	3	>1024	128	>1024	64	1024	16	512	8
129-8	129	Germany	No	4	>1024	>1024	>1024	1024	1024	64	512	64
151-1	151	Germany	No	3	>1024	>1024	>1024	1024	1024	256	512	32
157-4	157	Germany	No	1	>1024	>1024	>1024	512	1024	16	512	8
196-3	196	Germany	No	3	>1024	>1024	>1024	512	1024	512	256	64
198-4	198	Germany	No	3	>1024	>1024	>1024	1024	>1024	256	256	32
AN1	RD	UK	Yes	4	>1024	1024	>1024	512	>1024	512	128	16
116	LSM74	Belgium	No data	2	>1024	>1024	1024	128	1024	1024	512	128
170	HNF68	Belgium	No data	4	>1024	>1024	>1024	256	1024	64	512	32
251	DMF72	Belgium	No data	4	>1024	1024	>1024	256	1024	64	512	32
Mutation: C2598G												
Control strain: PAO1-pMES-23S(C2598G)					>1024	1024	512	256	64	32	32	16
198-1	198	Germany	No	4	>1024	>1024	>1024	512	>1024	64	128	4
BY3	CC	UK (LES)	Yes	1	>1024	1024	256	64	256	128	32	32
Double mutation: A2046G and C2598G												
129-8	129	Germany	No	4	>1024	>1024	>1024	1024	1024	64	>512	64

LES: Liverpool Epidemic Strain.



Origin	Medium	Clarithromycin		Azithromycin		Telithromycin		Solithromycin	
		MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
CF	CA-MHB	>1024	>1024	512	>1024	256	>1024	128	512
	RPMI-1640	>1024	>1024	128	>1024	64	1024	16	256
HAP	CA-MHB	>1024	>1024	256	512	64	128	32	64
	RPMI-1640	256	512	32	64	8	16	8	16

FIGURE 1 Minimum inhibitory concentration (MIC) cumulative distributions of macrolides and ketolides in RPMI-1640 and cation-adjusted Mueller–Hinton broth (CA-MHB) for a–c) cystic fibrosis (CF) and d–f) hospital-acquired pneumonia (HAP) isolates. CLR: clarithromycin; AZM: azithromycin; SOL: solithromycin. a) Clarithromycin and b) azithromycin were studied in the whole CF collection. A subset of the collection was then selected to cover the whole range of azithromycin MICs as measured in RPMI-1640 and used to evaluate ketolide MICs. c) The MIC distribution for this subset is compared for azithromycin and solithromycin. The number of isolates included in the study is indicated in each panel. The table below the graphs shows MIC_{50} and MIC_{90} values in both media for the subset of 76 CF isolates and the 48 HAP isolates (note that these values are 1 $\log_2$ dilution lower for clarithromycin in RPMI-1640 when considering the whole CF collection, but not different for azithromycin).

Outer membrane permeability

We showed that the increased susceptibility of *P. aeruginosa* to macrolides in RPMI-1640 is due to an increased permeability of the outer membrane in this medium [8]. We therefore investigated whether the lower susceptibility of CF *versus* HAP isolates could result from a reduced outer membrane permeability (figure 2). As anticipated, outer membrane permeability was higher in RPMI-1640 than in CA-MHB, but independently of the origin of the samples.

Efflux pump inhibition

We previously described that intrinsic resistance of *P. aeruginosa* to macrolides was in part attributable to constitutive expression of efflux pumps [8]. We therefore examined the influence of the efflux pump inhibitor PAβN on MICs. PAβN had limited effect on azithromycin activity against CF isolates (1 $\log_2$ dilution decrease in MICs for isolates with baseline values of 64 or 128 $mg{\cdot}L^{-1}$; no effect on other isolates;

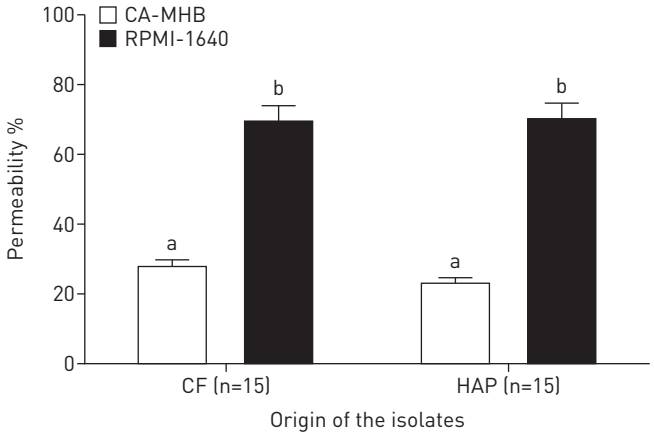


FIGURE 2 Outer membrane permeability of cystic fibrosis (CF) and hospital-acquired pneumonia (HAP) isolates in cation-adjusted Mueller–Hinton broth (CA-MHB) and RPMI-1640. Bacteria were incubated for 4 h in CA-MHB or RPMI-1640 medium and then with 25 µM 1-*N*-phenylnaphthylamine. Data are expressed as a percentage of the maximum value recorded in the presence of 3% Triton X-100 and are the mean±SD of the values recorded for the different isolates studied. Statistical analysis: two-way ANOVA with Tukey's multiple comparison test (data with different letters are significantly different from one another, $p<0.001$). The number of isolates included in the study is indicated on the *x*-axis.

figure 3a and b), indicating that increased efflux does not account for higher resistance in these isolates. Conversely, PAβN decreased MICs of 2–4 $\log_2$ dilutions for HAP isolates, independently of the baseline value (figure 3c and d), designating efflux as the main resistance mechanism in these isolates.

Sequencing of domain V of 23S rRNA

In Gram-positive bacteria, high-level resistance to macrolides is mediated by mutations in their ribosomal target. We therefore sequenced domain V of 23S rRNA for all isolates presented in figure 1c and e. Mutations were detected in 33 out of the 76 sequenced CF isolates (43%), while no mutations were detected in the 48 HAP isolates. In CF isolates, six different mutations were observed (A2045G, A2045T, A2046G, A2046T, C2598T and C2598G) at three specific positions and in one to four alleles (table 1).

Effect of mutations on macrolide and ketolide activity

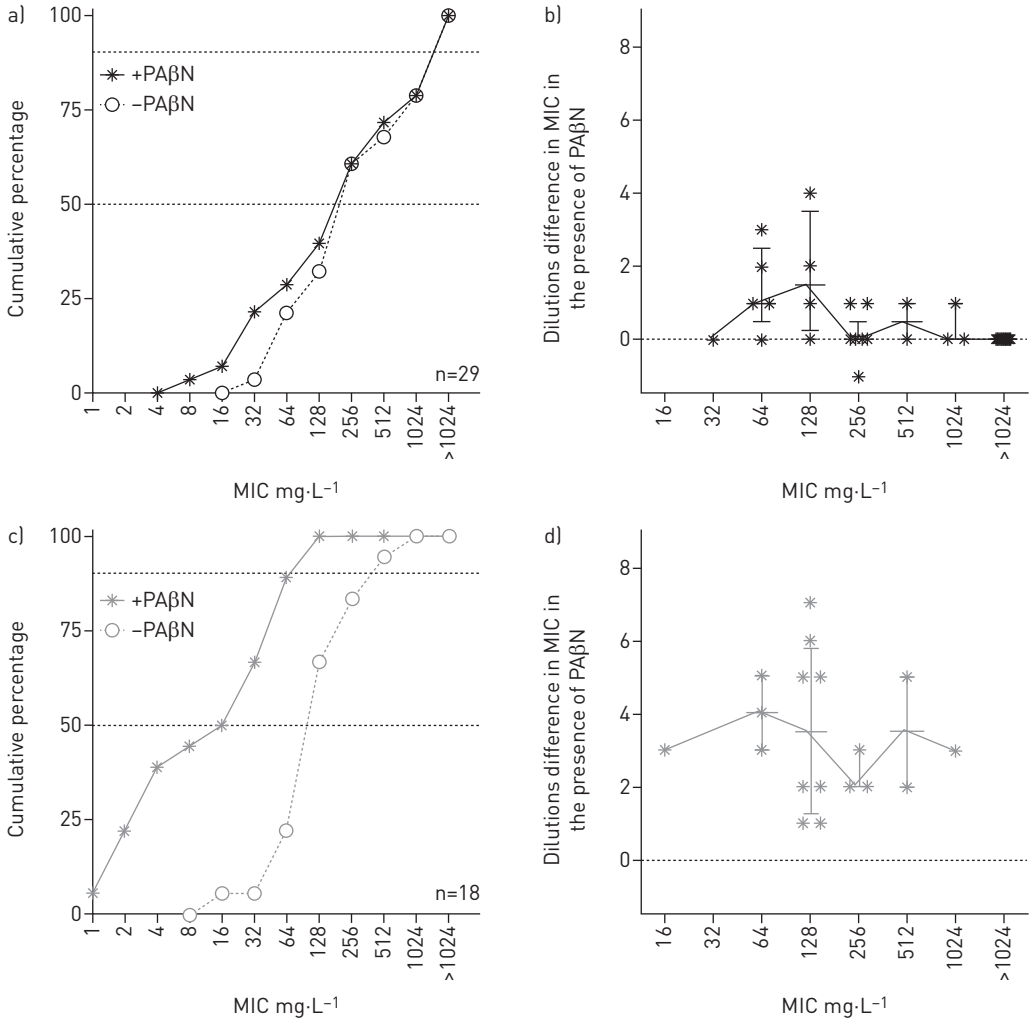
The MIC distributions of azithromycin and solithromycin in isolates showing mutations in domain V of 23S rRNA were compared with those of nonmutated isolates (figure 4). As expected, mutated isolates (figure 4a and b) were less susceptible than nonmutated isolates (figure 4c and d). Interestingly, the MIC distribution of nonmutated isolates from CF patients was superimposable to that of HAP isolates for both drugs and in both media (figure 4c and d), suggesting that mutations in part of the CF collection were responsible for the lower susceptibility observed in figure 1.

MICs for individual mutated isolates are shown in table 1 and in figure 4e and f, ordered according to the number of mutated alleles. MICs were in general more elevated in both media against isolates with mutations at position 2045 or 2046 than at position 2598, as well as in isolates with three or four mutated alleles. Solithromycin MICs remained low mainly in isolates mutated at position 2598, independently of the number of mutated alleles. One highly resistant isolate harboured two mutations in the four alleles.

To confirm the role of these mutations in resistance, PAO1 was transformed by plasmids containing each of the ribosomal mutations observed in clinical isolates. In these transformants, azithromycin MICs were higher than in PAO1, reaching 1024 mg·L^{-1} for those mutated in positions 2045 and 2046, and 256 mg·L^{-1} for those mutated in position 2598 (values in RPMI-1640). In contrast, ketolide MICs remained low regardless of media.

Additional studies in ketolide-resistant isolates

Some CF isolates showed reduced susceptibility to ketolides in RPMI-1640 (table 1), suggesting the presence of other resistance mechanisms. We therefore sequenced the genes encoding domain II of 23S rRNA and the ribosomal proteins L4 and L22 [24] in the 19 isolates with solithromycin MICs ⩾64 mg·L^{-1}, but no mutations were found. A cooperation with efflux could also be ruled out as MICs were not reduced by PAβN for most isolates (table 2). CF isolates are often mucoid and easily form biofilms with a matrix rich in alginate, DNA or proteins that prevents antibiotic access [25]. Solithromycin MICs were therefore also measured in the presence of enzymes degrading these polymeric substances (table 2). Alginate lyase,



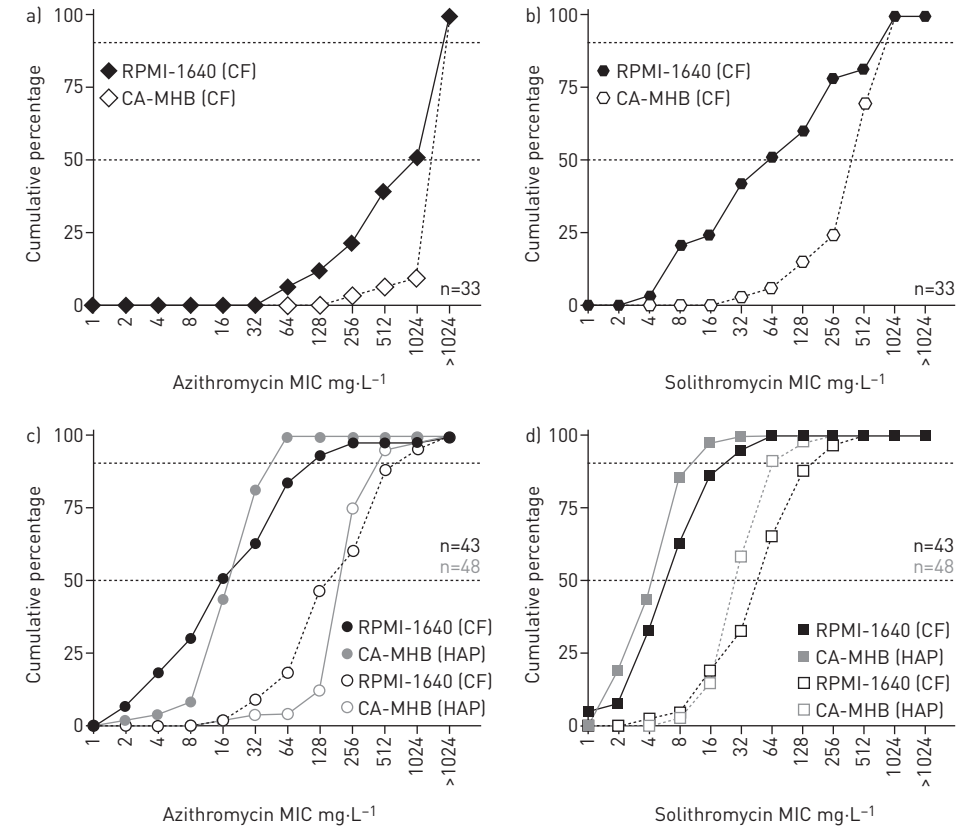
Origin	Medium	Azithromycin	
		MIC_{50}	MIC_{90}
CF	–PAβN	256	>1024
	+PAβN	256	>1024
HAP	–PAβN	128	>1024
	+PAβN	16	64

FIGURE 3 Effect of Phe-Arg-β-naphthylamide (PAβN) on azithromycin activity against a, b) cystic fibrosis (CF) and c, d) hospital-acquired pneumonia (HAP) isolates. Isolates were selected to cover the whole range of minimum inhibitory concentrations (MICs) in cation-adjusted Mueller–Hinton broth (CA-MHB). MICs were measured in CA-MHB in the presence (+) or absence (–) of 20 $mg \cdot L^{-1}$ PAβN and 1 mM $MgSO_4$. a, c) MIC cumulative distributions (the dotted lines indicate 50% and 90% values). b, d) Change in MIC (number of log_2 dilutions reduction) observed in the presence of PAβN for individual isolates classified according to their MIC in control conditions. Data are shown as individual values, with medians and interquartile range; the line joins median values. The table below the graphs shows MIC_{50} and MIC_{90} values in both conditions.

DNase or Proteinase K brought MICs back to values close to that measured for PAO1 in 14 isolates, among which those mutated in position 2598 were all reverted by alginate lyase.

Impact of azithromycin treatment on susceptibility to macrolides in CF isolates

In contrast to HAP patients, many CF patients are chronically exposed to macrolides, which might have contributed to resistance development. In the UK and German collections for which data on macrolide treatments were available, we compared azithromycin MICs in isolates from patients treated regularly by azithromycin *versus* those with no record of chronic azithromycin use (figure 5). In dot-plot analysis (figure 5b),



Origin	Ribosomal mutations	Medium	Azithromycin		Solithromycin	
			MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
CF	Yes	CA-MHB	1024	>1024	512	>1024
		RPMI-1640	1024	>1024	64	1024
	No	CA-MHB	256	1024	64	256
		RPMI-1640	16	128	8	32
HAP	No	CA-MHB	256	512	32	64
		RPMI-1640	32	64	8	16

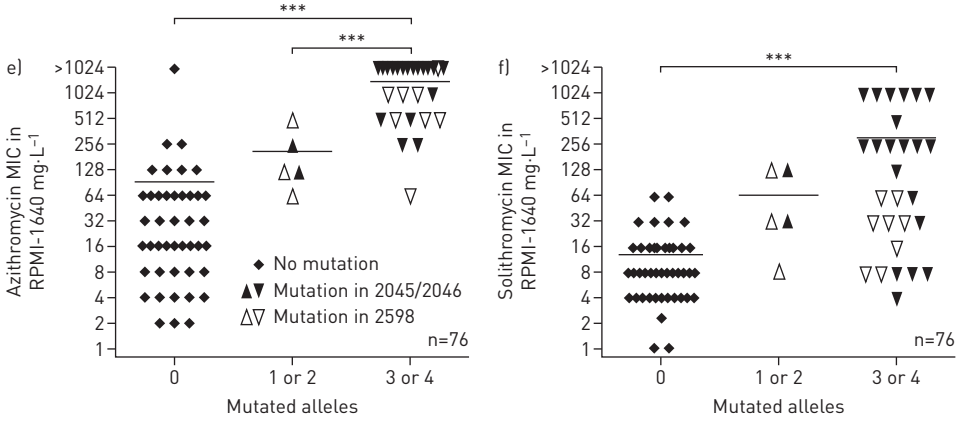


FIGURE 4 a–d) Minimum inhibitory concentration (MIC) cumulative distributions of a, c) azithromycin and b, d) solithromycin in cation-adjusted Mueller–Hinton broth (CA-MHB) and RPMI-1640 for cystic fibrosis (CF) and hospital-acquired pneumonia (HAP) isolates presenting a, b) mutations or c, d) no mutations in domain V of 23 S rRNA. e, f) relationship between the number of mutated alleles and the MIC: e) azithromycin; f) solithromycin. ***: p<0.001. The table shows MIC_{50} and MIC_{90} values in both media for mutated ("Yes") or nonmutated ("No") isolates. The number of isolates included in the study (same as those used in figure 1c and e) is indicated in each panel.

TABLE 2 Phenotype and solithromycin minimum inhibitory concentration (MICs) in RPMI-1640 for *Pseudomonas aeruginosa* PAO1 and mutated cystic fibrosis isolates with solithromycin MIC ⩾64 mg·L^{-1}, as measured in control conditions or in the presence of the efflux pump inhibitor Phe-Arg-β-naphthylamide (PAβN) or of enzymes degrading extracellular polymeric substances (alginate lyase, DNase or Proteinase K)

Strain	Mucoidy[¶]	Solithromycin MIC in RPMI-1640 mg·L^{-1}[#]				
		Control	+20 mg·L^{-1} PAβN and 1 mM $MgSO_4$	+20 U·mL^{-1} alginate lyase	+0.02% DNase and 20 mM $MgCl_2$	+100 mg·L^{-1} Proteinase K
PAO1	–	*16*	*16*	*16*	*16*	*16*
Mutation: A2045G						
2162	+	256	512	*16*	256	512
2964	+	512	512	512	*32*	512
3066	++	256	256	256	*64*	*16*
154-1	+	64	64	*8*	64	256
195-2	++	256	*16*	*16*	*32*	>512
CF5	++	>512	>512	256	256	256
BM6	++	256	256	256	256	>512
CF12	+	128	*16*	256	*32*	*32*
Mutation: A2045T						
BF4	++	>512	>512	>512	>512	>512
Mutation: A2046G						
2751	++	128	128	128	*32*	128
2801	++	256	256	256	*16*	>512
127-2	++	>512	>512	>512	>512	>512
134-2	+	256	256	256	*16*	*16*
205	++	>512	>512	>512	>512	>512
CF15	SCV[+]	>512	>512	>512	*8*	>512
CF45	+	>512	>512	>512	>512	>512
Mutation: C2598T						
196-3	++	64	128	*16*	64	128
116	++	128	64	*16*	128	512
Double mutation: A2046G and C2598G						
129-8	–	64	64	*16*	512	>512

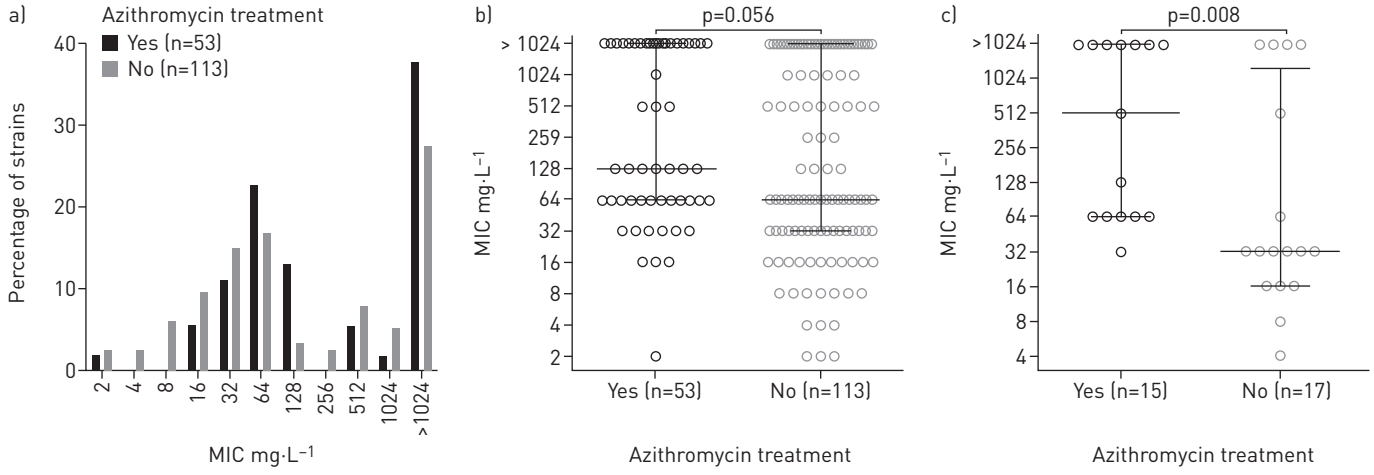
[#]: values in italics indicate MICs brought back to PAO1 levels±1 log_2 dilution when measured in the presence of the indicated agent; [¶]: mucoidy of the colony (–: nonmucoid phenotype; +: mucoid phenotype; ++: very mucoid phenotype) (see supplementary figure S1 for an example of each phenotype); [+]: small colony variant.

no significant difference but a trend to lower values in the median or geometric mean MIC was noticed for isolates collected from azithromycin-treated *versus* untreated patients. However, isolates with low MICs (⩽32 mg·L^{-1}) were more frequent in untreated patients (36%) than in azithromycin-treated patients (19%). Conversely, isolates with high MICs (>1024 mg·L^{-1}) were predominant in azithromycin-treated patients (38%) *versus* untreated patients (27%) (figure 5a). In LES clonal isolates (highly prevalent among CF patients in the UK [26]), MICs were significantly higher for those collected from azithromycin-treated patients (figure 5c). Among these clonal isolates, 39% (five out of 13) of those collected from treated patients showed ribosomal mutations *versus* 12% (two out of 17) only of those originating from patients who were not reported as chronically treated by macrolides (p=0.04, Chi-squared test).

Discussion

This study is, to the best of our knowledge, the first to describe the presence of macrolide resistance mechanisms in a large collection of *P. aeruginosa* from CF patients. It was facilitated by the use of RPMI-1640, in which macrolide MICs are considerably lower than in conventional broth [8]. Approximately half of the sequenced CF isolates did harbour high MICs and ribosomal mutations that were not observed in HAP isolates.

The low MICs observed for HAP isolates in RPMI-1640 are clearly due to the reduced expression of constitutive efflux transporters (as originally described for laboratory strains and a few clinical isolates [8]). Indeed, MIC distributions for these isolates are similar in RPMI-1640 *versus* CA-MHB supplemented by PAβN. However, this is not the case for CF isolates, for which PAβN only reduces the MIC of the most susceptible isolates, suggesting the selective expression of other specific resistance mechanisms.



	Azithromycin treatment		χ^2 p-value
	Yes	No	
MIC ≤32 mg·L^{-1}	10/53	41/113	0.01
MIC >1024 mg·L^{-1}	20/53	31/113	0.08

	Azithromycin treatment	
	Yes	No
Median	128 (64–2048)	64 (64–512)
Geometric mean	246 (146–416)	141 (95–210)

	Azithromycin treatment	
	Yes	No
Median	512 (64–2048)	32 (16–512)
Geometric mean	370 (140–979)	74 (25–225)

FIGURE 5 a) Minimum inhibitory concentration (MIC) distribution and b, c) scatter plots of MICs of azithromycin determined in RPMI-1640 for cystic fibrosis isolates from the UK and German collections stratified according to azithromycin treatment: a, b) whole UK and German collections; c) Liverpool Epidemic Strain clone only. "Yes": patients chronically treated by azithromycin; "No": patients not chronically treated by azithromycin. b, c) Data are shown as individual values with medians and interquartile ranges. The number of isolates included in the study is indicated on the *x*-axis; additional analyses are shown in the tables below the graphs (with 95% confidence intervals in brackets). Statistical analysis: a) p-values for Chi-squared (χ^2) analysis of specified MIC ranges given in the table below the graph; b, c) Mann–Whitney test.

As CF isolates with nonmutated rRNA have an MIC distribution similar to that of HAP isolates, we concluded that macrolide resistance in CF isolates is due to mutations in domain V of 23S rRNA. The detected mutations are located in three previously described positions [11], but we also found three additional nucleotide changes. Mutations in these positions confer macrolide resistance in other bacterial species [22] and we confirm using specific mutants that they also increase macrolide MICs in *P. aeruginosa*. Notably, mutations in positions 2045 and 2046 confer higher resistance than those in position 2598, probably because the first two positions are part of the macrolide-binding site, while the third position rather alters the conformation of the binding site [14]. We also show that resistance levels are globally higher in isolates harbouring mutations in several alleles of the corresponding gene.

Taken together, these findings led us to evaluate the effects of ketolides against CF isolates, as these antibiotics also bind to domain II of 23S rRNA [14]. As expected, CF isolates are more susceptible to ketolides than to macrolides, with solithromycin being more potent than telithromycin, as in other bacterial species [16]. While most of the isolates showing the A2046T or C2598T mutations had low MICs to solithromycin, those harbouring A2045G, A2045T or A2046G mutations generally had higher MICs, in the absence of mutations in domain II of 23S rRNA or ribosomal protein L4 or L22. This resistance could be related to the production of extracellular polymeric substances such as alginate, DNA or proteins, presumably preventing antibiotic penetration.

Importantly, we observed a trend to higher MICs in isolates from azithromycin-treated patients. Specifically in patients infected by the LES clone, mutated isolates with high MICs were more frequently identified from azithromycin-treated patients. This is of concern, as this clone is recognised as multiresistant [27]. Thus, macrolide resistance may, possibly, be more easily selected in the LES background, even though it is acquired by chromosomal mutation and not by gene transfer. Hypermutator variants have been detected, but infrequently in this clone (5–15%) [28, 29]. Cross-infection between patients treated in the same centre is described [18, 30, 31], especially for epidemic clones [32–34], but it is unlikely to markedly contribute to spread resistance here, as mutations are less frequent in LES isolates from nontreated patients.

Macrolides are not given to CF patients for their antibacterial properties against *P. aeruginosa*. Prescribers may, therefore, consider their use as innocuous with respect to resistance in this organism. Yet, we argue that the highlighted mutations are of concern. First, they may compromise some of the macrolide antivirulence effects that are directly or indirectly dependent on their binding to ribosomes and subsequent protein synthesis inhibition [2, 35]. Second, they may create a reservoir of resistance, possibly compromising the

activity of future drugs acting on the same target. In a broader context, the low but persisting serum concentrations of macrolides (especially azithromycin) may aggravate the risk of resistance development, as suggested for Gram-positive pathogens [36]. Providing high, local concentrations in the lung could help mitigate this risk. In this context, liposomes [37] or formulations for inhalation [38] are currently in development for macrolides. Further studies are also needed to define the potential benefit of fluoroketolides such as solithromycin that accumulate in the epithelial lining fluid and show potent anti-inflammatory properties [39, 40].

In the current clinical context, our findings may also be of immediate interest for clinicians in their dialogue with microbiologists. For clinicians, we raise the question of the long-term risk/benefit ratio associated with the widespread strategy of chronic use of oral macrolides in CF patients. For microbiologists, we provide an easy phenotypic method (*i.e.* testing activity of macrolides in RPMI-1640), applicable on a wide scale, to detect acquired resistance in CF samples. Prospective, comparative clinical trials to further document the impact of macrolide and ketolide exposure of CF patients for selection and spreading of resistance are therefore warranted.

Acknowledgements

We thank Cempra Pharmaceuticals (Chapel Hill, NC, USA) and R.L. Marvig (Technical University of Denmark, Lyngby, Denmark) for the kind gift of solithromycin and of the PAO1-pMES-23S(A2045G) strain, respectively. J. Turnidge (The University of Adelaide, Adelaide, Australia) gave useful advice for statistical analyses.

References

1 Cystic Fibrosis Foundation. Patient registry – annual data report. 2015. www.cff.org/Our-Research/CF-Patient-Registry/2015-Patient-Registry-Annual-Data-Report.pdf Date last accessed: February 25, 2017.
2 Imperi F, Leoni L, Visca P. Antivirulence activity of azithromycin in *Pseudomonas aeruginosa*. *Front Microbiol* 2014; 5: 178.
3 Zarogoulidis P, Papanas N, Kioumis I, *et al.* Macrolides: from *in vitro* anti-inflammatory and immunomodulatory properties to clinical practice in respiratory diseases. *Eur J Clin Pharmacol* 2012; 68: 479–503.
4 Southern KW, Barker PM, Solis-Moya A, *et al.* Macrolide antibiotics for cystic fibrosis. *Cochrane Database Syst Rev* 2012; 11: CD002203.
5 Clinical and Laboratory Standards Institute. Performance Standards for Antimicrobial Susceptibility Testing; 25th Informational Supplement. CLSI document M100-S25. Wayne, CLSI, 2015.
6 Masuda N, Sakagawa E, Ohya S, *et al.* Substrate specificities of MexAB–OprM, MexCD–OprJ, and MexXY–OprM efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2000; 44: 3322–3327.
7 Schweizer HP. Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. *Genet Mol Res* 2003; 2: 48–62.
8 Buyck JM, Plesiat P, Traore H, *et al.* Increased susceptibility of *Pseudomonas aeruginosa* to macrolides and ketolides in eukaryotic cell culture media and biological fluids due to decreased expression of *oprM* and increased outer-membrane permeability. *Clin Infect Dis* 2012; 55: 534–542.
9 Leclercq R. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. *Clin Infect Dis* 2002; 34: 482–492.
10 Nakajima Y. Mechanisms of bacterial resistance to macrolide antibiotics. *J Infect Chemother* 1999; 5: 61–74.
11 Marvig RL, Sondergaard MS, Damkiaer S, *et al.* Mutations in 23S rRNA confer resistance against azithromycin in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2012; 56: 4519–4521.
12 Riou M, Carbonnelle S, Avrain L, *et al. In vivo* development of antimicrobial resistance in *Pseudomonas aeruginosa* strains isolated from the lower respiratory tract of Intensive Care Unit patients with nosocomial pneumonia and receiving antipseudomonal therapy. *Int J Antimicrob Agents* 2010; 36: 513–522.
13 Van Bambeke F. Renaissance of antibiotics against difficult infections: focus on oritavancin and new ketolides and quinolones. *Ann Med* 2014; 46: 512–529.
14 Douthwaite S, Hansen LH, Mauvais P. Macrolide–ketolide inhibition of MLS-resistant ribosomes is improved by alternative drug interaction with domain II of 23S rRNA. *Mol Microbiol* 2000; 36: 183–193.
15 Llano-Sotelo B, Dunkle J, Klepacki D, *et al.* Binding and action of CEM-101, a new fluoroketolide antibiotic that inhibits protein synthesis. *Antimicrob Agents Chemother* 2010; 54: 4961–4970.
16 Van Bambeke F, Tulkens PM. The role of solithromycin in the management of bacterial community-acquired pneumonia. *Expert Rev Anti Infect Ther* 2016; 14: 311–324.
17 Barrera CM, Mykietiuk A, Metev H, *et al.* Efficacy and safety of oral solithromycin versus oral moxifloxacin for treatment of community-acquired bacterial pneumonia: a global, double-blind, multicentre, randomised, active-controlled, non-inferiority trial (SOLITAIRE-ORAL). *Lancet Infect Dis* 2016; 16: 421–430.
18 Cheng K, Smyth RL, Govan JR, *et al.* Spread of beta-lactam-resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. *Lancet* 1996; 348: 639–642.
19 Mustafa M-H, Chalhoub H, Denis O, *et al.* Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients through Northern Europe. *Antimicrob Agents Chemother* 2016; 60: 6735–6741.
20 Lamers RP, Cavallari JF, Burrows LL. The efflux inhibitor phenylalanine–arginine beta-naphthylamide (PAbetaN) permeabilizes the outer membrane of Gram-negative bacteria. *PLoS One* 2013; 8: e60666.
21 Loh B, Grant C, Hancock RE. Use of the fluorescent probe 1-*N*-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 1984; 26: 546–551.
22 Vester B, Douthwaite S. Macrolide resistance conferred by base substitutions in 23S rRNA. *Antimicrob Agents Chemother* 2001; 45: 1–12.
23 Gutell RR, Larsen N, Woese CR. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol Rev* 1994; 58: 10–26.

24 Hisanaga T, Hoban DJ, Zhanel GG. Mechanisms of resistance to telithromycin in *Streptococcus pneumoniae*. *J Antimicrob Chemother* 2005; 56: 447–450.
25 Mann EE, Wozniak DJ. *Pseudomonas* biofilm matrix composition and niche biology. *FEMS Microbiol Rev* 2012; 36: 893–916.
26 Winstanley C, Langille MG, Fothergill JL, *et al.* Newly introduced genomic prophage islands are critical determinants of *in vivo* competitiveness in the Liverpool Epidemic Strain of *Pseudomonas aeruginosa*. *Genome Res* 2009; 19: 12–23.
27 Ashish A, Shaw M, Winstanley C, *et al.* Increasing resistance of the Liverpool Epidemic Strain (LES) of *Pseudomonas aeruginosa* (Psa) to antibiotics in cystic fibrosis (CF) – a cause for concern? *J Cyst Fibros* 2012; 11: 173–179.
28 Lopez-Causape C, Rojo-Molinero E, Mulet X, *et al.* Clonal dissemination, emergence of mutator lineages and antibiotic resistance evolution in *Pseudomonas aeruginosa* cystic fibrosis chronic lung infection. *PLoS One* 2013; 8: e71001.
29 Fothergill JL, Mowat E, Ledson MJ, *et al.* Fluctuations in phenotypes and genotypes within populations of *Pseudomonas aeruginosa* in the cystic fibrosis lung during pulmonary exacerbations. *J Med Microbiol* 2010; 59: 472–481.
30 Milczewska J, Wolkowicz T, Zacharczuk K, *et al.* Cross-infections with *Pseudomonas aeruginosa* in patients with cystic fibrosis attending the Warsaw Centre. *Dev Period Med* 2015; 19: 60–65.
31 Pitt TL. Cross infection of cystic fibrosis patients with *Pseudomonas aeruginosa*. *Thorax* 2002; 57: 921.
32 Ramsey BW. To cohort or not to cohort: how transmissible is *Pseudomonas aeruginosa*? *Am J Respir Crit Care Med* 2002; 166: 906–907.
33 O'Malley CA. Infection control in cystic fibrosis: cohorting, cross-contamination, and the respiratory therapist. *Respir Care* 2009; 54: 641–657.
34 Saiman L, Siegel J. Infection control in cystic fibrosis. *Clin Microbiol Rev* 2004; 17: 57–71.
35 Tan H, Zhang L, Weng Y, *et al.* PA3297 counteracts antimicrobial effects of azithromycin in *Pseudomonas aeruginosa*. *Front Microbiol* 2016; 7: 317.
36 Lynch JP III, Martinez FJ. Clinical relevance of macrolide-resistant *Streptococcus pneumoniae* for community-acquired pneumonia. *Clin Infect Dis* 2002; 34: Suppl. 1, S27–S46.
37 Solleti VS, Alhariri M, Halwani M, *et al.* Antimicrobial properties of liposomal azithromycin for *Pseudomonas* infections in cystic fibrosis patients. *J Antimicrob Chemother* 2015; 70: 784–796.
38 Siekmeier R, Hofmann T, Scheuch G. Inhalation of macrolides: a novel approach to treatment of pulmonary infections. *Adv Exp Med Biol* 2015; 839: 13–24.
39 Kobayashi Y, Wada H, Rossios C, *et al.* A novel macrolide solithromycin exerts superior anti-inflammatory effect via NF-kappaB inhibition. *J Pharmacol Exp Ther* 2013; 345: 76–84.
40 Ribeiro C, Martino M, Brighton B, *et al.* Anti-inflammatory effects of macrolides in a model relevant to CF airway epithelial inflammation. *Pediatr Pulmonol* 2011; 46: Suppl. 34, 289 (poster 219).

TABLE S1: Primers used in this study

Name	Sequence
Forward-Domain V-23S	5'-GGTGCCGGAAGGTTAATTGATG-3'
Reverse-Domain V-23S	5'-GCAGCCCCTCTCAAATCTCAAAC-3'
Reverse-Domain V-23S for Allele 1*	5'- CCCTTCATCGGAAGCCATCT-3'
Reverse-Domain V-23S for Allele 2*	5'-GAGTGGAATCGCCCGTCTTG-3'
Reverse-Domain V-23S for Allele 3*	5'-CACGGACATCGGTATCGGAC-3'
Reverse-Domain V-23S for Allele 4*	5'-GCTCCAGCCTTTCACGGAG-3'
Forward-Domain II-23S	5'-AGAGGGGAGTGAAATAGAACCT-3'
Reverse-Domain II-23S	5'-GTCGATTAACGTTGCGCAGG-3'
Forward-L4 protein	5'-GAACGCAATCTGCTGCTGGT-3'
Reverse-L4 protein	5'-AAACGAATTGGCTCTTGCCG-3'
Forward-L22 protein	5'-GGCCACAAACTGGGCGAGTT-3'
Reverse-L22 protein	5'-GGTCTGCGTACCAAACGGA-3'

*****to amplify the four alleles of rRNA operon of *P. aeruginosa* corresponding to the following locus tag of the gene 23S rRNA based on complete genome sequence of PAO1 (www.pseudomonas.com): PA0668.4, PA4280.2, PA4690.2, and PA5369.2 for alleles 1, 2, 3 and 4 respectively.

FIGURE S1

Representative images of different colony morphotypes: A: non-mucoid, B: SCV, C: mucoid and D: very mucoid.